PERSISTENT, VERTICAL-MIGRATION RHYTHMS IN BENTHIC MICROFLORA. VI. THE TIDAL AND DIURNAL NATURE OF THE RHYTHM IN THE DIATOM HANTZSCHIA VIRGATA [1]

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During ebb tide in certain intertidal mud and sand-flats, irregular areas of the exposed substratum become green or golden brown in color. With the return of the flooding tide—or often just prior to its return—the color fades and disappears. Microscopic examination of these sediments reveals that the color may be due to a superficial accumulation of enormous numbers of protozoans, small metazoans, or more commonly, single-celled algae. These organisms dwell in the sediments during tidal inundation and move up onto the surface sands during tidal exposure —a behavior pattern called a vertical-migration rhythm. Dinoflagellates (Herdman, 1924), euglenoids (Bracher, 1919; Palmer and Round, 1965), a chrysomonad (Fauré-Fremiet, 1950), several species of diatoms (Fauvel and Bohn, 1907; Aleem, 1950; Callame and Debyser, 1954; Round and Palmer, 1966), and a zooxanthellae-containing planarian (Gamble and Keeble, 1903) are all known to undergo these tide-associated rhythms in vertical migration. At times these organisms are present in such great numbers that one investigator (Herdman, 1924, p. 59) observed that ". . . the diatoms were so abundant on the surface that their photosynthetic activity was distinctly *audible* as a gentle sizzling . . . and the sand was frothy with bubbles of gas, presumably oxygen given off by them."

The rhythmic behavior of a few of these organisms has been studied in the laboratory and found to persist in natural day-night conditions but in the absence of the tide (Fauvel and Bohn, 1907; Bracher, 1919; Herdman, 1924; Fauré-Fremiet, 1950, 1951), and in constant conditions, *i.e.*, constant temperature, continuous illumination of a constant intensity, and no tides (Palmer and Round, 1965; Round and Palmer, 1966). Our studies (*loc. cit.*) have revealed an interesting and unexpected aspect of vertical-migration rhythms, namely, that in constant conditions the rhythms of two species of *Euglena* and eight species of diatoms are *diurnal*, rather than *tidal*, *i.e*, the 24.8-hour period of the rhythm—as displayed in nature—is not expressed in the laboratory; instead, the persistent rhythm has a 24-hour period. These data, combined with certain field studies by other investigators (*e.g.*, Perkins, 1960), suggest that possibly all overt tidal vertical-migration rhythms might actually represent underlying 24-hour rhythms which are entrained and thus transformed by the tides in nature. Alone among the modern studies in contradiction of this supposition, is the work of Fauré-Fremiet (1951) on the vertical-migration rhythm of the diatom *Hantzschia am-*

phioxys. He reports (p. 173) that when *Hantzschia*-bearing sand samples were returned to the laboratory and ". . . exposed to diffuse light from the window, on succeeding days (the longest period of observation being six days), the [*Hantz-schia*] re-appeared on the surface of the sand at the same time as low tide in their natural habitat." He used only the color change of the sand as an indication of whether or not the cells were on the surface and stresses the lack of precision of this type of observation. Because his work stands as an exception to our original hypothesis, it has stimulated us to re-examine the vertical migratory behavior of this organism in greater detail and using quantitative methods. Both field and laboratory studies were carried out.

This diatom inhabits the intertidal sand-flats of Barnstable Harbor, Cape Cod, Mass., and was previously identified in the paper of Fauré-Fremiet (1951) as *H. amphioxys.* We have compared the diatom with collections held at the Philadelphia Academy of Sciences and the British Museum and find that it is *H. virgata* var. *intermedia* (Grun.).[2] During the summer months it tends to be the dominant species of an algal community containing the diatoms *Amphora, Navicula, Amphiprora, Pleurosigma,* and *Nitzschia;* the dinoflagellate *Amphidinium;* the euglenoids, *Euglena* and *Trachelomonas;* and the cyanophyceans *Chroococcus, Merismopedia* and *Oscillatoria.* Preliminary studies indicate that all these subdominants also undergo vertical-migration rhythms in the field and the laboratory.

Method

In order to obtain quantitative estimates of cell concentrations on the surface sediments at any one time, a method previously described in the literature (Palmer and Round, 1965) was employed. In brief, just as the ebbing tide uncovered the sampling station, numerous small pieces (9 mm.^{2}) of ordinary microscope-lens-cleaning tissue were placed on the sediment surface. The *Hantzschia,* in their migratory ascent to daylight, moved up through the sediments and into the interstices of the paper. Tissues were then periodically removed from the sediment during tidal exposure, the diatoms washed out in a drop of water on a microscope slide, and their numbers counted. Replicate samples were taken and averaged.

In order to collect cells for study in the laboratory, 10-mm. lengths of glass tubing (35 mm. in diameter) were inserted into the sediment and removed with a core of *Hantzschia*-bearing sand within them. These cores, still retained within the glass rings, were placed in small Petri dishes and returned to the Marine Biological Laboratory at Woods Hole, Mass., where water was added to the moat-like space between the outside of the glass ring and the inner wall of the Petri dish. The samples were kept in Precision Scientific Incubators at a constant temperature of 18° C. and overhead illumination of 110 foot-candles from Westinghouse, 15-watt, cool-white fluorescent tubes. The cells were maintained in alternating light-dark photoperiods (symbolized at L:D) with the light on between 0530 and 2000 hours (the approximate time between sunrise and sunset), or in continuous illumination (L:L). It should be pointed out, however, that inherent in vertical-migration rhythms is a periodic sojourn beneath the sand surface, placing the organisms into

[2] We wish to thank Dr. R. Patrick and Mr. R. Ross for their help and the loan of type material.

semi-darkness.   This obviously tends to negate the desired effect of the overhead experimental light regime.   The laboratory populations were also sampled with the lens-paper technique and the average of 3–6 samples used for each cell count. Because this technique unavoidably reduces the size of the sample populations, during long-term observations, sampling on some days was intentionally omitted.

RESULTS

Field observations

The sampling station on the Barnstable Harbor sand-flats is uncovered by the tide for an average duration of 4.5 hours once every 12.4 hours.   Field observations were made at intervals during the summers of 1965 and 1966 and were timed so that the presence of the cells on the surface during morning, midday, and evening low tides could be described and compared.

It was found that when low tide straddles the time of sunrise the cells do not appear on the surface until shortly after the time of sunrise.   Once they begin to appear, their numbers rapidly increase to a maximum value which then remains relatively constant until about 30–60 minutes before the return of high tide, at which time the cells begin to re-burrow back into the substratum.   A representative curve is described in Figure 1.
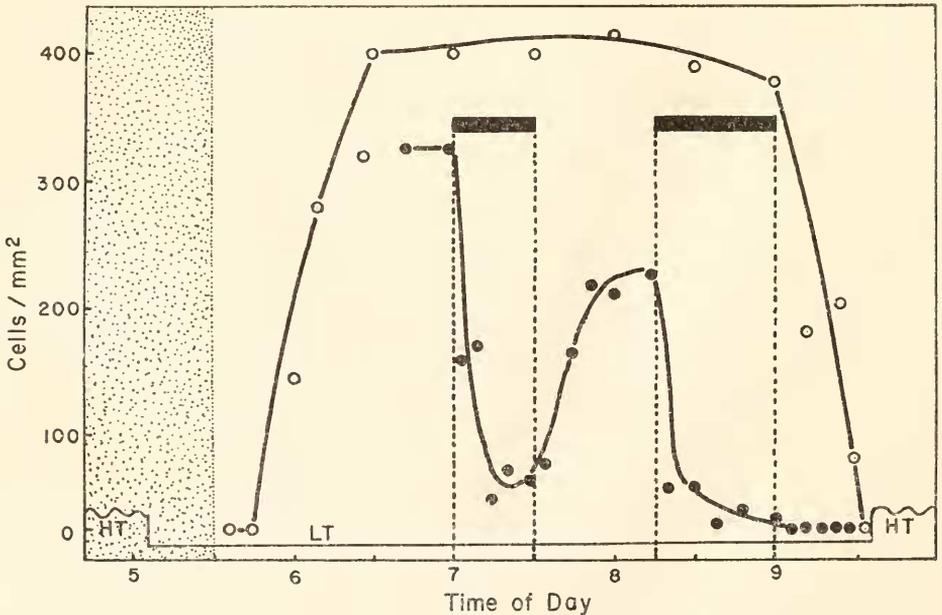

FIGURE 1.   Field observations of the vertical-migration rhythm in *Hantzschia*.   Wavy lines (HT) represent the times of high tide.   Straight line (LT) subtending and connecting consecutive wavy lines indicates time of low tide.   The time of sunrise is represented by the boundary between stippled and unstippled areas.   The shaded horizontal bars supported by dashed lines signify periods when the sands were covered by opaque canisters.   See text for further description.

During midday exposures, the cells begin to appear on the surface 15–30 minutes after the tidal water recedes, increase in number to a fairly constant density which is maintained for about three hours, and then commence to re-burrow about 30 to 60 minutes in advance of the incoming tide. (Re-burrowing in *anticipation* of actual return of flood tide is a common feature of vertical-migration rhythms [Callame and Debyser, 1954; Palmer and Round, 1965; Round and Palmer, 1966].) By the time of inundation only the remnants of the densest patches remain on the surface and these cells re-burrow within 15 minutes after being submerged. A small fraction of the cells is often washed away. Measurements of the sediment water content during a tidal exposure showed that on a bright, windy day, the water content drops as much as 14% below the value obtained just after exposure. Up to one hour before the actual reflooding of the area—and simultaneous with the time the cells begin to re-burrow—the interstitial water content of the sand begins to increase steadily. This may well be the stimulus which initiates re-burrowing in apparent anticipation of the return of high water. As will be shown later, however, re-burrowing is under the control of a biological clock and the cells need no external stimulus if this kind to re-burrow.

As late afternoon low tides approach the time of sunset, the diatoms do not remain on the surface for the duration of low water, but instead re-burrow slightly before sunset. When the sand flats are first exposed at 1630 hours, or later, the cells never appear on the surface.

The diatoms never appear on the surface at night and they can be kept from emerging on the surface during daylight by artificially darkening the sediments with opaque canisters. Similarly, cells already on the surface can be made to re-burrow by artificial darkening. This is seen in Figure 1, where the cells were artificially darkened between 0700 and 0730 and again between 0845 and 0900. After the first darkening the cells returned to the surface; after the second, they did not, but at this time the rest of the population was also in the process of re-burrowing. The upper curve in this figure represents untreated cells in an adjacent patch and thus acts as a control for the darkening experiments.

Laboratory studies

The rhythmic behavior of *Hantzschia* was first studied in constant light and temperature. Under these conditions the rhythm was found to persist for as long as eleven days. A representative experiment is seen in Figure 2. While night-time values were not determined for this particular set of data, numerous other all-night observations have adequately demonstrated that the cells never appear on the surface at night, even when samples are maintained in L:L. The approximate times of low tide in nature are indicated for each day, and show that the cells in the laboratory appear on the surface in approximate synchrony with those in Barnstable Harbor—strongly suggesting that the rhythm is actually tidal. However, the possibility exists that the rhythm may actually be one with a fundamental period of 24 hours, which—as is common with most persistent rhythms—has become circadian in constant conditions, and by chance, has a period of 24.8 hours. To test this possibility, samples were placed in L:D, a condition which restricts solar-day rhythms to their fundamental 24-hour period. Figure 2 shows that the cells continue to appear on the surface later each day and again in approximate synchrony
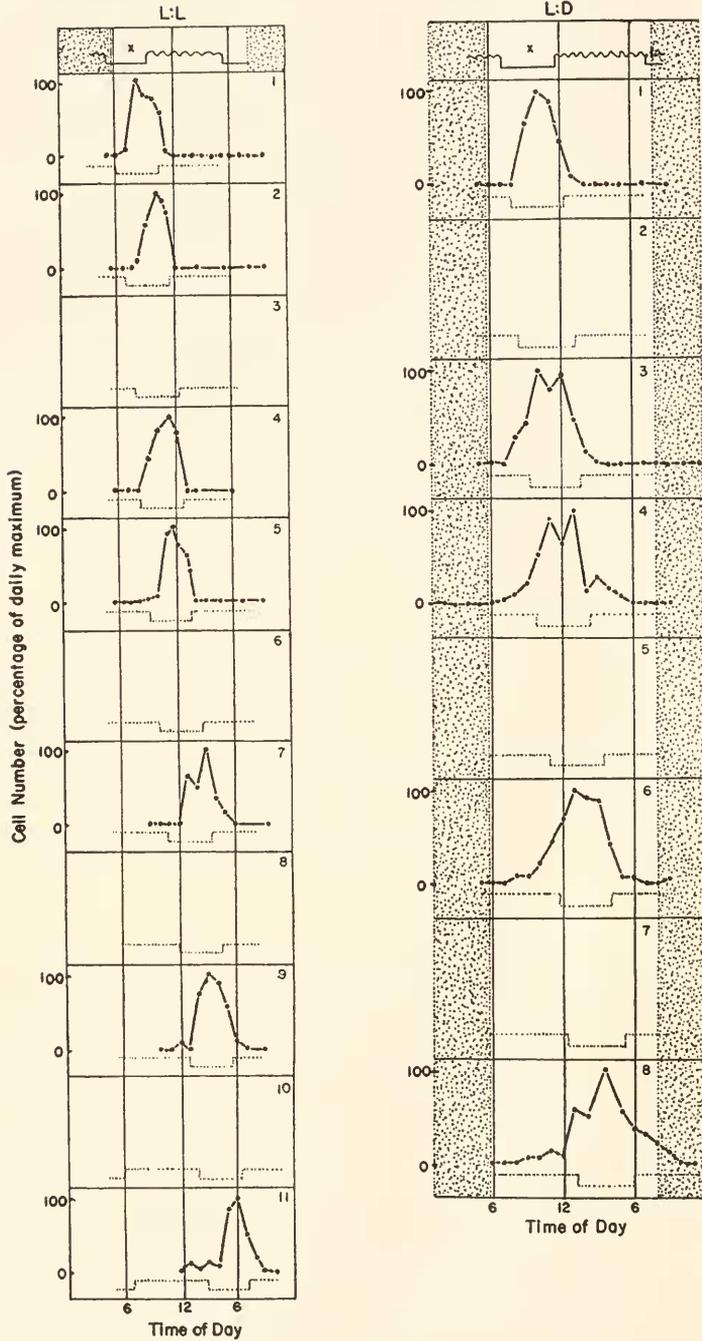
FIGURE 2.

with the tidal exposure in nature. The persistent rhythm is indeed a lunar-day rhythm.

The 12.4-hour interval between successive low tides is such that in the summer, when an afternoon low tide approaches the time of sunset, the following morning low tide begins to overlap with sunrise. Therefore, twice each month, there are a few days when the sand flats are exposed to sunlight twice a day: once in the morning and again in the afternoon. As the afternoon exposure approaches dusk, the diatoms abandon this phase of the rhythm and now appear on the surface during the early morning low tide. This rather drastic change in phase was studied in the laboratory.

Cells were collected during a mid-afternoon low tide and placed in L:D in the incubators. Sampling began the next day and, as seen in Figure 3, the cells came up strongly in the late afternoon. On the second day the afternoon peak virtually collapsed, and by the third day the cells now appeared in the morning. The same observations were carried out in L:L and, quite remarkably, the same change in phase occurred. Both the observations in L:D and L:L have been repeated several times with the same results.

DISCUSSION

Clock control of the rhythm

Persistent, tidal rhythms have been previously described for a variety of physiological functions, *e.g.*, oxygen consumption in crabs (Brown *et al.*, 1954) and snails (Sandeen *et al.*, 1954) ; spontaneous locomotor activity in crabs (Bennett *et al.*, 1957; Naylor, 1958; Palmer, 1966), amphipods (Enright, 1962; Morgan, 1965), and fish (Gibson, 1965), and in filtration rate in mussels (Rao, 1954). These rhythms may be described as tidal rhythms (*i.e.*, rhythms with periods of 12.4 hours), or better, as *bimodal* (or *biphasic*) lunar-day rhythms with periods of 24.8 hours. When studied in relation to a 24-hour-day scale, the dual peaks of the lunar-day rhythm advance at a rate of 50 minutes/cycle across the solar day.

The overt lunar-day rhythm in *Hantzschia* differs considerably from the above rhythms in two major ways. First, the rhythm is *unimodal, i.e.*, the cells appear on the surface only once every 24.8 hours. Secondly, the single maximum scans across the hours corresponding to daylight at a tidal rate of 50 minutes/day and then, in a matter of just a few days, rephases back to the morning hours again. Any model derived to explain the rhythm in *Hantzschia* must take into account these two unique properties of the rhythm.

The curves obtained in L:D, shown in Figure 3, indicate that when the supra-surface phase of the rhythm reaches the dark portion of the imposed photoperiod the rhythm rephases to the morning hours, suggesting that the times of "light off"

FIGURE 2. Persistence of the vertical-migration rhythm in constant light (L:L) and in alternating light-dark period (L:D). In both conditions the rhythm displays a period of about 24.8 hours. Consecutive days run from top to bottom. Stippling indicates dark periods. X = time of collection of samples. State of tide on day of collection symbolized as in Figure 1. Depressions in dotted lines represent times of low tide in nature on days when rhythm was studied in the laboratory. For ease in comparison the data are expressed in percentages (the highest cell count in each cycle was designated as 100 and all other values as percentages of this). In no case was 100% less than 2.9×10^3 cells/cm.^{2}.

and "light on" act as guideposts for the extreme phase relationships of the rhythm. By way of analogy, the scanning movements of the single peak across the day can be likened to the movement of a typewriter carriage, which slowly and systematically—one letter at a time—moves across the instrument to the far carriage stop, and is then rapidly swept back to the starting margin to begin another journey. The carriage stops, which dictate the extent of movement of the carriage, could be
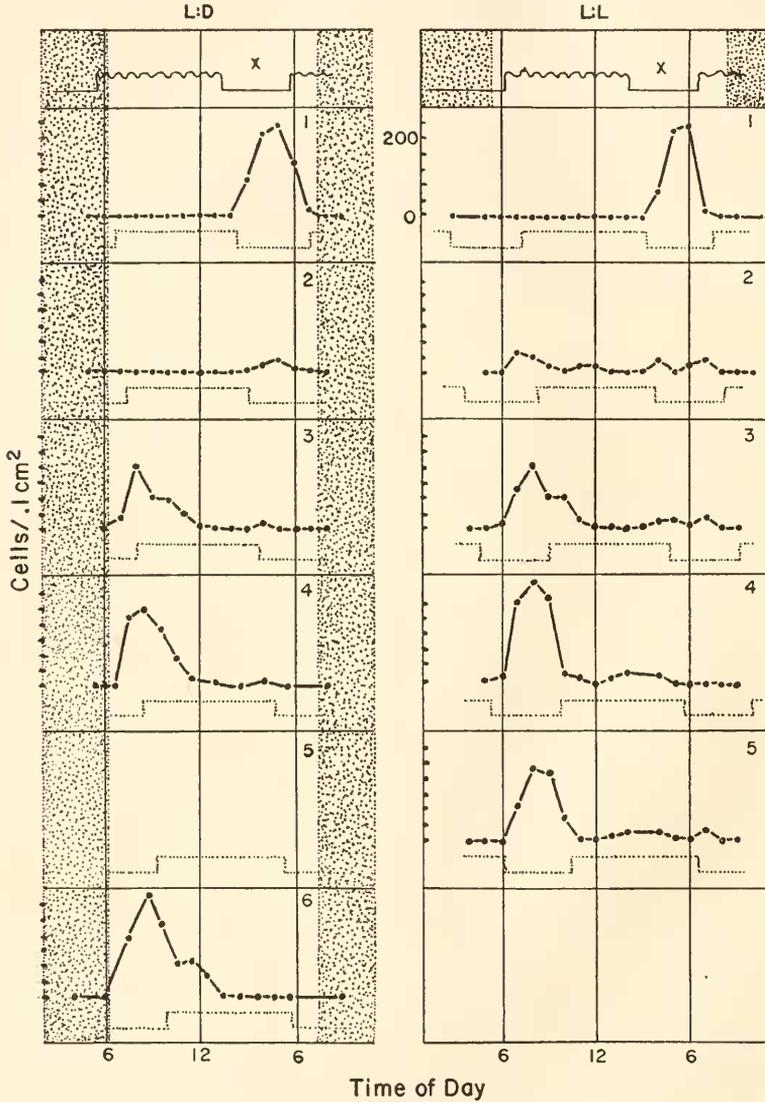

FIGURE 3. The phase change of the persistent vertical-migration rhythm in alternating light-dark periods (L:D) and in constant light (L:L). Symbols the same as Figure 2. Ordinate scale for all days given in upper right hand column.
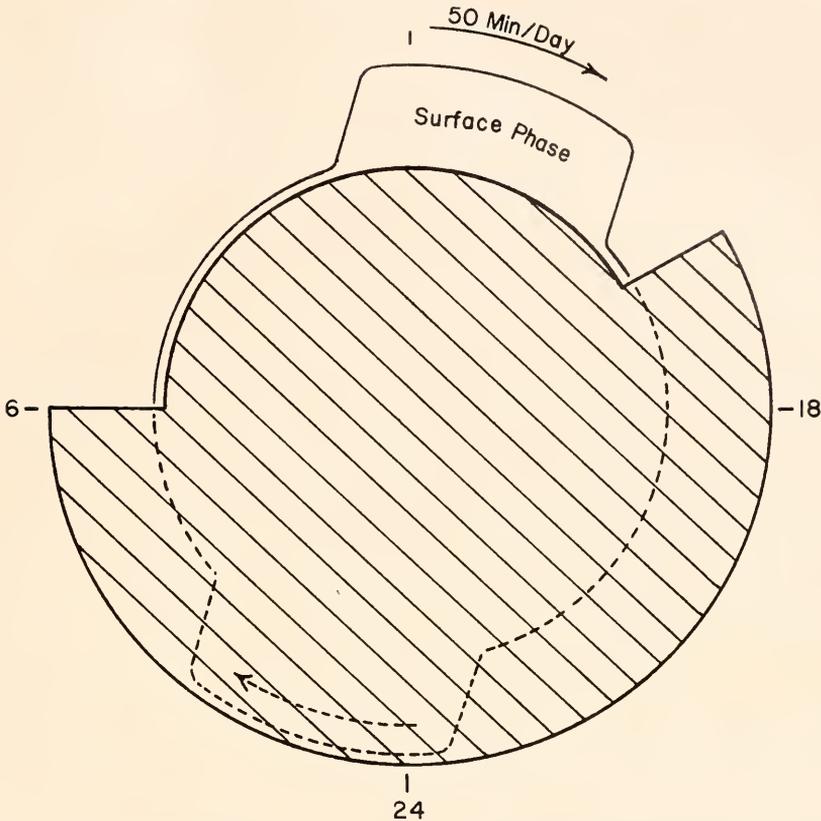
FIGURE 4. Diagrammatic representation of the interaction of a 24.8-hour bimodal vertical-migration rhythm (here represented as a disk with opposing bulges; each bulge signifying the surface phase of the rhythm) and a 24-hour suppression-expression rhythm (represented as an incomplete disk superimposed over the lunar-day rhythm). The shaded area of the disk is that part of the 24-hour rhythm that suppresses the night-time phase of the migration rhythm, and the open segment the part that allows the expression of the daytime supra-surface phase. Because the supra-surface phase of the lunar rhythm occurs 50 minutes later each day it eventually falls under the influence of the suppressive portion of the solar-day rhythm. As this phase is inhibited the unexpressed early morning phase is expressed. The net result is an *apparent* rephase of the migration rhythm.

likened to the times of "light on" and "light off" in the rephasing of the rhythm. However, the analogy breaks down when the rhythm is re-examined in L:L as no such obvious "stops" were then present, yet the same apparent rephase took place. As a consequence, we prefer to adopt a working hypothesis based on the presence of two interacting clock systems. One is a lunar-day clock which, of course, measures periods of 24.8 hours. This clock controls a vertical-migration rhythm characterized by *two* supra-surface phases, 12.4 hours apart. This rhythm is represented diagrammatically in Figure 4, by a rotating disk with diametrically opposed, conspicuous bulges. Each of these lateral protrusions represents a supra-surface phase of the rhythm and therefore each has a width equal to 4.5 hours. Coupled to

the lunar-day clock is a solar-day clock which measures periods of 24 hours. This horologue controls a rhythm which is characterized by two alternating phases: one which suppresses the night-time supra-surface phase of the bimodal vertical-migration rhythm, and a second phase which permits the expression of the migration rhythm in the daytime. The action of this rhythm is represented in Figure 4, by a partially shaded stationary disk superimposed over the disk representing the lunar-day rhythm. The shaded section represents the suppressive role of this rhythm and the open segment or "window" represents the portion in which the tidal rhythm is not inhibited. The size of the "window" was determined by field observations of the hours of daylight during which the cells appeared on the surface.

By means of such a dual mechanism, as the expressed phase of the lunar-day rhythm occurs progressively later each day (50 minutes/day), it eventually coincides with the suppressive phase of the solar-day clock and is inhibited. Concurrent with this event, the opposite peak of the migration rhythm moves into the "window" and is now expressed. The net result is an *apparent* rephase of the rhythm from afternoon to morning hours.

The feasibility of such a hypothesis is strengthened by the studies of Naylor (1958), Barnwell (1963), Chandrashekaran (1965), and Palmer (1966), which all demonstrate that it is a very common feature for organisms which display persistent lunar-day rhythms to have a solar-day clock system associated with, and modifying, the lunar rhythms. Enright's conclusion (1963), that a single organismic process does not have simultaneous tidal and solar-day components, has now been shown to be premature; it was based on his interpretation of earlier work (Bennett *et al.,* 1957; Naylor, 1958) and by his own work on an organism which possessed only a tidal rhythm (Enright, 1962). Actually, in intertidal organisms, the co-existence of solar-day and lunar-day components in a particular rhythmic function is a commonly encountered pattern.

Role of light

The importance of light in the expression of the vertical-migration rhythm of *Hantzschia* manifests itself in a variety of ways: (1) the cells never appear on the surface during night-time low tides; and when low tides straddle the time of light and darkness, the cells appear on the surface only during the illuminated portion of the tidal exposure, (2) cells may be prevented from appearing on the surface by artificial darkening, and (3) cells already on the surface can be made to reburrow by artificial darkening. It is therefore quite apparent that light is necessary to bring the cells to the surface and to hold them there for some critical time. However, light does not always have an attractive effect, for in L:L in the laboratory, the cells do not come to the surface during the times corresponding to night, or daytime high tides. Reasoning deductively, this certainly indicates that the *Hantzschia* must undergo a rhythmic change in responsiveness to light and this rhythm must be of fundamental importance in their migratory behavior. While no systematic studies have yet been made on the existence of a persistent tidal rhythm in phototaxis in *Hantzschia,* field observations by Fauré-Fremiet (1951) and Palmer (1960) have demonstrated a sign reversal in the phototactic response of this diatom during the supra-surface phase of its rhythm. The organisms were

found to respond positively to light during the initial and mid-portions of their stay on the sediment surface and then become indifferent or negative to light just before the return of the tide. A persistent rhythm in phototaxis is known for another unicell, *Euglena* (Pohl, 1948).

Other environmental factors also contribute to the migratory movements of the diatom. Inundation by high tide water is of paramount importance in the expression of the rhythm (Palmer, 1960), and the fact that the diatoms reburrow when artificially darkened (burrowing being a specific *directional* movement) indicates that geotactic orientation must also be important.

Adaptive nature of the rhythm

It has been tacitly assumed by past investigators that a vertical-migration rhythm represents a highly adaptive relationship with the environment. It is supposed that these sand-dwelling organisms move out onto the surface in order to undergo maximum photosynthetic activity during the daytime, and then re-burrow to avoid being washed away by the returning tide (Ganapati *et al.*, 1959). Certainly some of the non-conformers who do not re-burrow before the flooding tide sweeps over them are often seen to be washed away, thus supporting the latter half of the above contention. However, Taylor and Palmer (1963) have described the photosynthetic light-saturation curve for the benthic microflora community on Barnstable— *Hantzschia*, of course, being a prominent member—and these results demonstrate that sufficient light penetrates through the upper 1.5 mm. of sediment to enable the cells to photosynthesize at above 90% of their maximum capacity. Full sunlight is well above the optimum and actually inhibits photosynthesis somewhat. Quite clearly, then, it is unnecessary for the cells to "risk" a journey onto the surface— and the possibility of being washed away—in order to undergo efficient photosynthesis. It may be that the response is just primarily a phototactic one, the adaptive significance of which is less obvious.

SUMMARY

1. The diatom, *Hantzschia virgata*, appears on the surface sands of Barnstable Harbor, Mass., during *daytime* low tides. Surface accumulations of this organism reach such concentrations that the sand takes on a golden-brown color. As the tide returns the cells re-burrow into the sand.

2. The cells can be prevented from emerging onto the surface sands at low tide by artificially darkening the area with an opaque covering just as the tide recedes. Cells already on the surface can be made to re-burrow by similarly placing them in darkness.

3. The vertical-migration rhythm will persist in the laboratory in constant illumination, constant temperature, and away from the influence of the tide for as long as eleven days. During this time the cells remain in approximate synchrony with the feral cells in nature.

4. In nature, when the times of low tide approach sunset, the cells rephase their rhythm to the early morning hours of daylight. Cells collected during late afternoon low tides and returned to L:D or L:L in the laboratory, undergo a similar rephasing in an interval of just three days.

5. To explain the various unique properties of this rhythm, it is postulated that the rhythm is a manifestation of an interacting dual-clock system: a lunar-day clock which measures periods of 24.8 hours and is responsible for a bimodal migration rhythm; and a solar-day clock responsible for the suppression of the night-time supra-surface phase of the migration rhythm.

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